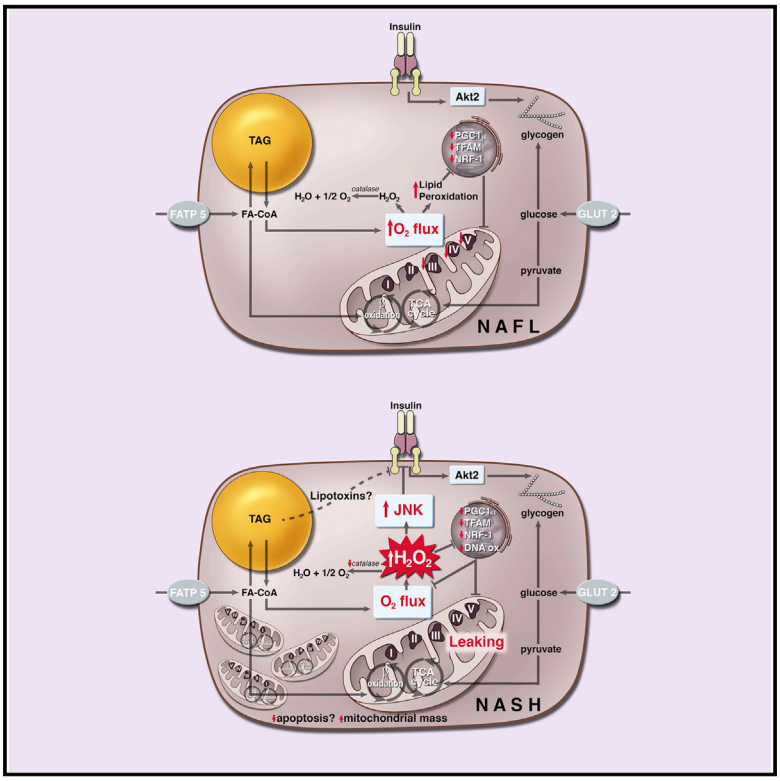
Short Article

Cell Metabolism

Adaptation of Hepatic Mitochondrial Function in Humans with Non-Alcoholic Fatty Liver Is Lost in Steatohepatitis

Graphical Abstract



Highlights

- Direct measurement of hepatic mitochondrial content and capacity in humans
- Upregulated hepatic respiration in obese humans with and without NAFL
- Impaired respiratory capacity and proton leakage in obese humans with NASH
- Elevated oxidative stress coupled to reduced anti-oxidant capacity in NASH

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In Brief

Koliaki et al. show that obese individuals without steatohepatitis (NASH) have increased mitochondrial respiratory rates compared to lean ones, suggesting hepatic mitochondrial flexibility at early stages of obesity-related insulin resistance. This adaptation is, however, lost in patients with NASH, who have lower maximal respiration, despite higher mitochondrial mass.

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Adaptation of Hepatic Mitochondrial Function in Humans with Non-Alcoholic Fatty Liver Is Lost in Steatohepatitis

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SUMMARY

The association of hepatic mitochondrial function with insulin resistance and non-alcoholic fatty liver (NAFL) or steatohepatitis (NASH) remains unclear. This study applied high-resolution respirometry to directly quantify mitochondrial respiration in liver biopsies of obese insulin-resistant humans without (n = 18) or with (n = 16) histologically proven NAFL or with NASH (n = 7) compared to lean individuals (n = 12). Despite similar mitochondrial content, obese humans with or without NAFL had 4.3- to 5.0-fold higher maximal respiration rates in isolated mitochondria than lean persons. NASH patients featured higher mitochondrial mass, but 31%–40% lower maximal respiration, which associated with greater hepatic insulin resistance, mitochondrial uncoupling, and leaking activity. In NASH, augmented hepatic oxidative stress (H_2O_2, lipid peroxides) and oxidative DNA damage (8-OH-deoxyguanosine) was paralleled by reduced anti-oxidant defense capacity and increased inflammatory response. These data suggest adaptation of the liver ("hepatic mitochondrial flexibility") at early stages of obesity-related insulin resistance, which is subsequently lost in NASH.

INTRODUCTION

Liver is a key organ involved in energy homeostasis and pathogenesis of type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). NAFLD comprises steatosis (non-alcoholic fatty liver [NAFL]), steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma. NAFLD associates with both hepatic and whole-body insulin resistance, suggesting common pathogenic mechanisms (Roden, 2006).

Mitochondria orchestrate energy metabolism by substrate oxidation via β-oxidation, tricarboxylic acid cycle (TCA), adenosine triphosphate (ATP) synthesis through oxidative phosphorylation (OXPHOS), and reactive oxygen species (ROS) formation (Murphy, 2009). In skeletal muscle, insulin resistance may coexist with mitochondrial alterations such as lower mitochondrial density, OXPHOS gene expression, and ATP synthesis (Mootha et al., 2003; Morino et al., 2005; Szendroedi et al., 2007).

Whether similar mitochondrial alterations are also present in livers of insulin-resistant humans remains unclear. Previous studies applied only indirect methods to assess hepatic mitochondria (Koliaki and Roden, 2013). While there is some evidence for impaired hepatic mitochondrial function in T2DM (Schmid et al., 2011, Szendroedi et al., 2009) and NASH (Pérez-Carreras et al., 2003; Sanyal et al., 2001), Sunny et al. reported increased hepatic mitochondrial function in obese humans with NAFLD using stable isotope techniques (Sunny et al., 2011). The latter study could neither account for mitochondrial content nor differentiate between NAFL and NASH. No data on simultaneous direct measurement of OXPHOS capacity and mitochondrial density are available in livers of humans at early stages of insulin resistance such as obesity and NAFL.

Thus, we established in humans ex vivo high-resolution respirometry (HRR) to quantify hepatic mitochondrial respiration and combined it with measures of mitochondrial content. We analyzed intraoperative liver samples from obese humans without NAFL (OBE NAFL−), with NAFL (OBE NAFL+), with NASH (OBE NASH), and lean humans without NAFLD (CON). Our data suggest differential regulation of hepatic mitochondrial respiration in obese insulin-resistant humans with and without NASH.

RESULTS

Obese Humans with NASH Have Markedly Reduced Hepatic Insulin Sensitivity

The groups did not differ in age, gender distribution, and fasting free fatty acids (Table 1). All obese groups had higher body mass and resting energy expenditure than CON, but body mass did not differ among obese groups. OBE NASH presented with

Table 1. Participants' Characteristics

	CON	OBE NAFL−	OBE NAFL+	OBE NASH
Age	41 ± 3 years	39 ± 3 years	41 ± 3 years	51 ± 3 years
Gender (male/female), n	5/7	3/15	2/14	3/4
Body mass index	25.5 ± 0.7 kg/m^2	48.3 ± 1.9 kg/m^2[a]	53.7 ± 2.1 kg/m^2[a]	47.3 ± 0.7 kg/m^2[a]
Fasting glucose	78 ± 1 mg/dl	88 ± 3 mg/dl	87 ± 3 mg/dl	127 ± 19 mg/dl[a,b,c]
M value	7.7 ± 0.8 mg/kg/min	3.5 ± 0.3 mg/kg/min[a]	2.5 ± 0.3 mg/kg/min[a]	1.4 ± 0.9 mg/kg/min[a,b]
Rd	47 ± 8 μmol/kg/min	23 ± 3 μmol/kg/min[a]	14 ± 1 μmol/kg/min[a]	15 ± 4 μmol/kg/min[a]
EGP suppression	82% ± 3%	76% ± 4%	73% ± 7%	59% ± 18%[a,b]
HbA1c	5.3% ± 0.1%	5.5% ± 0.1%	5.5% ± 0.1%	6.6% ± 0.6%[a,b,c]
SGOT	21 ± 1 U/l	31 ± 4 U/l	32 ± 8 U/l	58 ± 13 U/l[a,b,c]
SGPT	24 ± 2 U/l	31 ± 13 U/l	39 ± 4 U/l	59 ± 6 U/l[a,b,c]
Fasting free fatty acids	528 ± 73 μmol/l	618 ± 51 μmol/l	623 ± 77 μmol/l	698 ± 52 μmol/l
Fasting triglycerides	99 ± 12 mg/dl	99 ± 10 mg/dl	147 ± 18 mg/dl	261 ± 76 mg/dl[a,b,c]
Resting energy expenditure	1,548 ± 68 kcal/day	2,096 ± 124 kcal/day[a]	2,233 ± 167 kcal/day[a]	2,080 ± 100 kcal/day[a]
Δ respiratory quotient (clamp-basal)	0.13 ± 0.01	0.11 ± 0.03	0.03 ± 0.01[a,b]	0.020 ± 0.003[a,b]
HCL	2.1% ± 1.0%	2.6% ± 0.5%	26.9% ± 3.7%[a,b]	70.7% ± 2.8%[a,b]
NAFLD score	0.6 ± 0.3	0.6 ± 0.2	2.9 ± 0.4[a,b]	6.9 ± 0.6[a,b]

Data are represented as means ± SEM. CON (n = 12) were compared to OBE humans classified into NAFL− (n = 18), NAFL+ (n = 16), and NASH (n = 7) based on liver histology (NAFL−: HCL < 5.56%, NAFLD score < 3, NAFL+: HCL ≥ 5.56%, NAFLD score < 3, NASH: NAFLD score ≥ 5). EGP, endogenous glucose production; HbA1c, glycosylated haemoglobin; HCL, hepatocellular lipids; NAFLD, non-alcoholic fatty liver disease; Rd, rate of glucose disposal; SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase.

[a] $p < 0.05$ versus CON

[b] $p < 0.05$ versus OBE NAFL−

[c] $p < 0.05$ versus OBE NAFL+

higher fasting glucose and triglycerides, glycosylated hemoglobin, and liver transaminases compared to other groups. Obese groups had markedly lower whole-body insulin sensitivity compared to CON, but only OBE NASH had lower hepatic insulin sensitivity. Both OBE NAFL+ and NASH exhibited reduced whole-body metabolic flexibility, assessed by indirect calorimetry, and higher liver fat content and NAFLD score compared to OBE NAFL− and CON.

Obese Humans without, but Not with NASH, Exhibit Upregulated Hepatic Mitochondrial Respiration

In liver tissue, maximal uncoupled respiration (state u) related to β-oxidation and TCA cycle activity was higher in both OBE NAFL− (+85%) and OBE NAFL+ (+50%) than in CON but lower in NASH (−51% versus OBE NAFL−, −40% versus OBE NAFL+, −10% versus CON) (Figure 1A). In isolated mitochondria, maximal uncoupled respiration related to TCA cycle activity was up to 4.3- to 5.0-fold higher in OBE NAFL− and NAFL+ than in CON, whereas the respective respiration rates were 31%–40% lower in NASH compared to both obese groups (Figure 1B). Similar differences were observed for state 3 respiration related to complex I (glutamate) or combined complex I and II activity (succinate). These alterations were seen in the presence of higher hepatic citrate synthase activity (CSA), reflecting larger mitochondrial mass in NASH compared to all other groups ($p < 0.001$) (Figure 1C). Expressing respiration rates per mitochondrial protein content yielded similar results (Table S1). Hepatic mitochondrial DNA content was not different among groups (copy numbers per cell; CON: 1,822 ± 188; OBE NAFL−: 1,752 ± 122; OBE NAFL+: 1,783 ± 122; OBE NASH: 1,700 ± 138).

To examine whether altered respiration associates with impaired coupling of substrate oxidation to ATP production as suggested in obese mice (Chavin et al., 1999) and NASH patients (Serviddio et al., 2008), we exposed isolated liver mitochondria to several substrates and oligomycin (o), an ATP synthase inhibitor revealing leak respiration, and assessed mitochondrial coupling efficiency from respiratory control ratio (RCR; state 3/state o) and leaking control ratio (LCR; state o/state u). RCR gradually declined across all groups from CON to NASH, suggesting mitochondrial uncoupling in obese humans (Figure 1D). This was aggravated in NASH patients, who exhibited markedly higher LCR than all other groups (Figure 1E).

To further investigate possible mechanisms underlying altered respiration in OBE groups, we measured hepatic mRNA expression of transcription factors regulating mitochondrial biogenesis and electron transport chain (ETC) complex protein expression. Expression of mitochondrial transcription factor A (*TFAM*), PPARγ-coactivator 1a (*PGC1a*), and nuclear respiratory factor 1 (*NRF-1*) was lower in both OBE NAFL+ and NASH compared to CON (Figures 1F–1H). Of note, expression of complexes III, IV, and V was lower in OBE NAFL+ than in CON (Figure S1). After correction for mitochondrial content, expression of complexes I, III, IV, and V was lower only in NASH than in CON and OBE NAFL−.

Only NASH Patients Present with Hepatic Oxidative DNA Damage

ROS production has been implicated in NAFLD progression (Pessayre, 2007). Indeed, all obese groups displayed elevated hepatic lipid peroxidation, assessed from thiobarbituric reactive substances (TBARS) (Figure 2A). Furthermore, OBE NASH

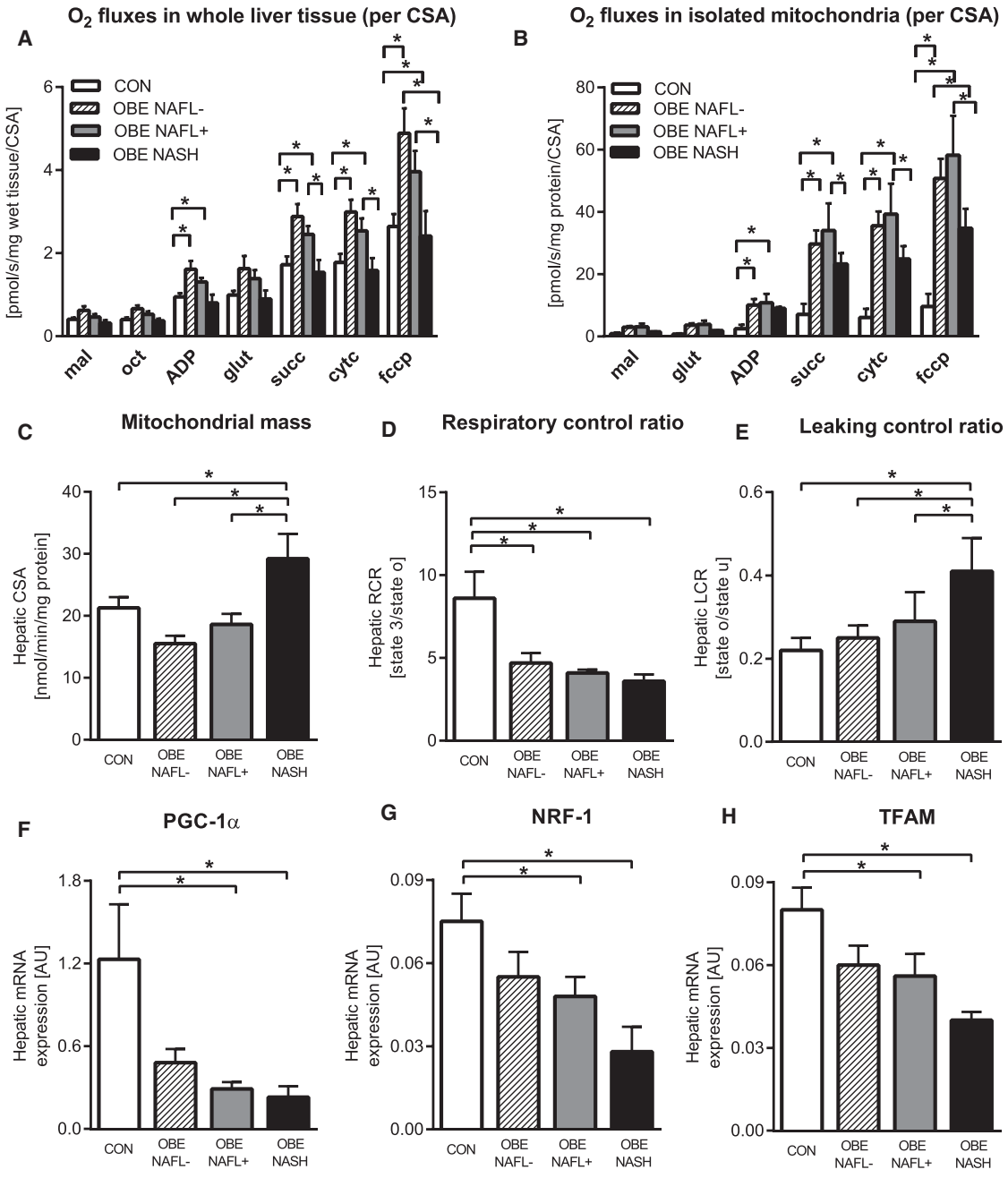

Figure 1. Measures of Hepatic Mitochondrial Function and Content in Lean, Obese Humans with and without Non-Alcoholic Fatty Liver and Obese with Non-Alcoholic Steatohepatitis

(A and B) O_2 fluxes (mean ± SEM; 12 CON, 18 OBE NAFL−, 16 OBE NAFL+, 7 OBE NASH) in (A) whole tissue and (B) isolated mitochondria upon adenosine diphosphate (ADP), cytochrome c (cyt c), substrates (mal, malate; oct, octanoyl-carnitine; glut, glutamate; succ, succinate), and carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) as uncoupling factor. All fluxes are normalized to citrate synthase activity (CSA).

(C) CSA as a measure of mitochondrial mass.

(D) Respiratory control ratio (RCR) defined as state 3/state o serving as marker of mitochondrial coupling.

(E) Leaking control ratio (LCR) defined as state o/state u as an index of proton leak.

(F–H) mRNA expression of regulators of mitochondrial biogenesis (F: PGC-1α, peroxisomal proliferator-activated receptor gamma coactivator 1α; G: NRF-1, nuclear respiratory factor 1; H: TFAM, mitochondrial transcriptional factor A).

CON, lean; OBE NAFL−, obese without non-alcoholic fatty liver; OBE NAFL+, obese with non-alcoholic fatty liver; OBE NASH, obese with non-alcoholic steatohepatitis.

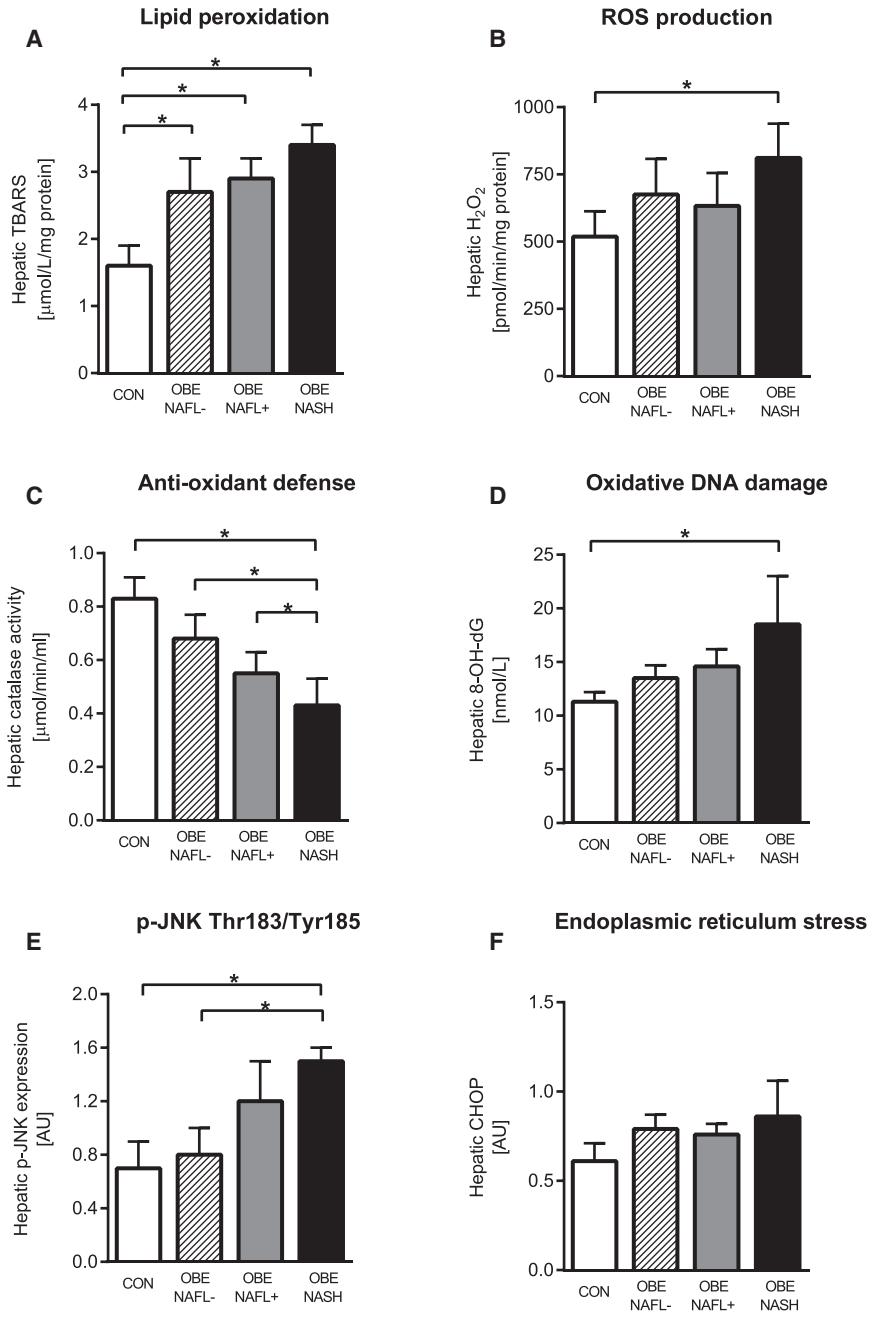

Figure 2. Oxidative Stress and Anti-Oxidant Capacity in Obese Humans with and without NASH

(A) Hepatic thiobarbituric reactive substances (TBARS), (B) hepatic hydrogen peroxide (H_2O_2), (C) hepatic catalase activity, (D) hepatic 8-OH-deoxyguanosine (8-OH-dG), (E) hepatic p-JNK Thr183/Tyr185 expression, and (F) hepatic CHOP expression. Data are represented as means ± SEM (n = 7–16/group), *p < 0.05.

showed greater mitochondrial H_2O_2 release after succinate and antimycin-A stimulation than CON, indicating augmented ROS production (Figure 2B). Hepatic catalase activity, reflecting anti-oxidant defense mechanisms, was unchanged in OBE NAFL− and NAFL+ but clearly impaired in NASH (Figure 1C). Hepatic 8-OH-deoxyguanosine (8-OH-dG), a marker of oxidative DNA damage, was increased only in NASH (Figure 2D).

Animal studies suggest concomitant induction of oxidative stress and inflammatory pathways, such as the c-Jun-N-terminal kinase (JNK)/NFκB, during development of hepatic insulin resistance (Satapati et al., 2012). In line with these data, NASH patients had elevated hepatic JNK phosphorylation at Thr183/Tyr185 (Figure 2E). The pro-apoptotic transcription factor CHOP, a marker of hepatic endoplasmic reticulum (ER) stress, tended to be higher only in NASH (p = 0.1) (Figure 2F).

NASH Patients Show Systemic Inflammation but Not Systemic Oxidative Stress

Circulating adipocytokines may also play a role in the pathogenesis of NAFLD (Cusi, 2012). Serum interleukin-1 receptor antagonist (IL1ra) was higher in both OBE NAFL+ and NASH compared to CON (Figure 3A), whereas serum interleukin-6 was elevated only in NASH (Figure 3B). Serum fibroblast growth factor 21 (FGF21) was higher in both OBE NAFL+ and NASH than in CON and OBE NAFL− (Figure 3C). High-molecular weight adiponectin was lower in all obese groups than in CON (Figure 3E). Serum TBARS, reflecting systemic oxidative stress, were not different between groups (Figure 3F).

Respiration Correlates Positively with Whole-Body Insulin Resistance and Hepatic Steatosis

Maximal uncoupled respiration related to TCA cycle activity in isolated mitochondria correlated positively with hepatic fat content (r = 0.476, p = 0.025) and plasma free fatty acids (r = 0.440, p = 0.028) and negatively with whole-body insulin sensitivity (r = −0.641, p = 0.001). Maximal uncoupled respiration in liver tissue correlated positively with fasting plasma glucose (r = 0.655, p < 0.001) and serum triglycerides (r = 0.390, p = 0.015). Across all groups, hepatic CSA correlated positively with NAFLD score (r = 0.593, p = 0.001), consistent with higher CSA in NASH. Whole-body insulin sensitivity correlated positively with serum adiponectin (r = 0.538, p = 0.001) and negatively with IL-6 (r = −0.468, p = 0.006) and hepatic lipid peroxidation (r = −0.426, p = 0.019). Hepatic insulin resistance correlated positively with serum IL-6 (r = 0.491, p = 0.005) and FGF21 (r = 0.585, p = 0.001).

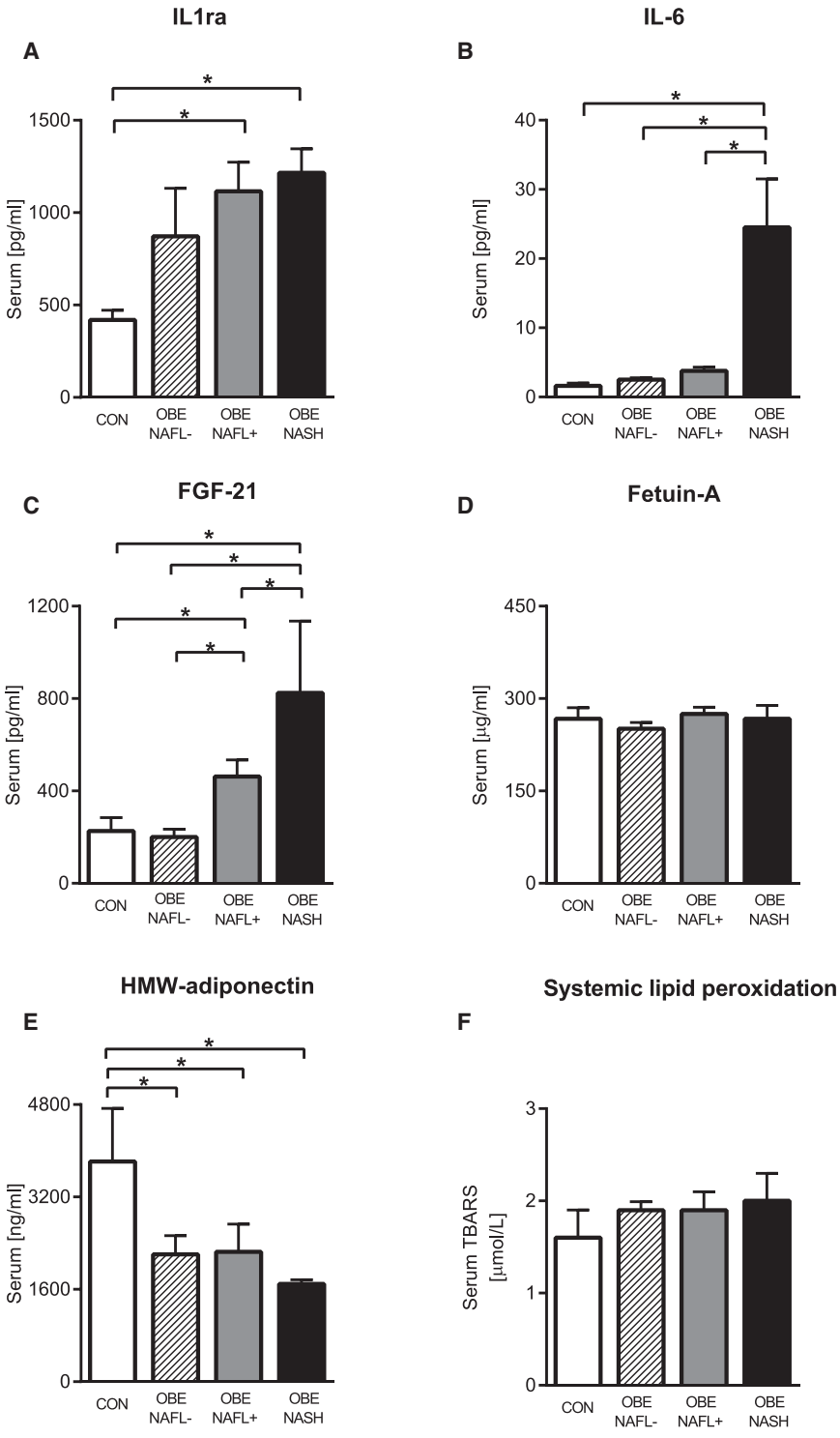

Figure 3. Circulating Markers of Inflammation and Oxidative Stress in Obese Humans with and without NASH

Serum concentrations of (A) interleukin-1 receptor antagonist (IL1ra), (B) interleukin-6 (IL-6), (C) fibroblast growth factor 21 (FGF21), (D) fetuin-A, (E) high molecular weight (HMW) adiponectin, and (F) thiobarbituric reactive substances (TBARS). Data are represented as means ± SEM (n = 7–17/group), *p < 0.05.

DISCUSSION

This study reports direct evidence for a compensatory upregulation of hepatic mitochondrial respiration in obese insulin-resistant humans with and without fatty liver. This adaptation is abolished in obese humans with NASH, who also exhibit greater hepatic insulin resistance, leaking mitochondria, hepatic oxidative stress, and systemic inflammation. These data suggest the operation of a mechanism of "hepatic metabolic flexibility," by which liver mitochondria adapt to altered bioenergetic demands preceding advanced NAFLD. Of note, all obese groups had a comparable degree of obesity so that the observed effects are not determined by whole-body obesity but rather by the degree of hepatic steatosis.

Obese humans with and without steatosis presented with higher O_2 flux rates both in liver tissue and isolated mitochondria compared to controls, in the context of comparable peripheral insulin resistance and normal hepatic insulin sensitivity. This is in line with the elevated hepatic mitochondrial activity observed in obese mouse models (Buchner et al., 2011). The greater respiratory capacity in OBE NAFL− and NAFL+ was not due to differences in mitochondrial content. In obese humans, Nair at al. found lower hepatic ATP content using ^{31}P-MRS (Nair et al., 2003) but provided no information on insulin resistance or presence of NAFLD. Reduced hepatic ATP may coexist with elevated respiration in the setting of mitochondrial uncoupling. Lower hepatic RCR of obese humans with and without NAFL in the current study provides evidence for decreased coupling and suggests that elevated respiration does not necessarily reflect efficient ETC. Inefficient ETC may be present in obese humans with NAFL, despite an upregulation of mitochondrial pathways upstream of respiration such as TCA cycle and β-oxidation (Sunny et al., 2011). In the present study, OBE NAFL− and NAFL+ featured similarly increased hepatic lipid peroxidation, likely due to augmented respiration and impaired coupling. Hepatic ROS production and oxidative DNA damage were not significantly elevated, which can result from their intact anti-oxidant defense capacity. Nevertheless, NAFL+ differed from NAFL− by lower hepatic expression of *PGC-1α*, *NRF1*, and *TFAM*, indicating

impaired mitochondrial biogenesis, as confirmed by lower ETC complex expression. These alterations may pave the way to NAFLD progression.

OBE NASH featured lower respiration along with reduced RCR. Mitochondrial uncoupling, already evident in OBE NAFL− and NAFL+, was aggravated in NASH, who had further elevated hepatic LCR, reflecting increased proton leak across mitochondrial membrane. This may explain the higher H_2O_2 emission from ETC, challenging the anti-oxidant defense capacity. Indeed, NASH patients had reduced hepatic catalase activity in line with the downregulated catalase expression reported in murine NASH models (Gornicka et al., 2011). In turn, the increased hepatic 8-OH-dG indicates hepatocellular oxidative damage in agreement with previous reports, suggesting enhanced nuclear expression of 8-OH-dG in hepatocytes of NASH patients (Seki et al., 2002). Interestingly, hepatic CSA was higher, while ETC complex expression tended to be lower in NASH. As a result, the ratio of ETC complex expression to CSA was even lower in NASH, indicating lower density of functional mitochondria, in line with reports of abnormal mitochondrial morphology in livers of NASH patients (Sanyal et al., 2001). The higher mitochondrial mass in NASH does not necessarily require increased mitochondrial biogenesis but may alternatively result from reduced degradation of damaged and dysfunctional mitochondria (Wang et al., 2015).

OBE NASH also exhibited higher circulating triglycerides and glucose, both of which may contribute to insulin resistance and abnormal energy metabolism. Hepatic lipid overload may have stimulated mitochondrial respiration, creating a pseudo-hypoxic state with induction of deleterious oxidative modifications of mitochondrial proteins (Mantena et al., 2009), promoting impaired respiration. On the other hand, patients with T2DM and hepatic insulin resistance exhibit both lower in vivo measured hepatic ATP content (Szendroedi et al., 2009) and flux through ATP synthase (Schmid et al., 2011), pointing to a possible role of circulating glucose. In the present study, hepatic insulin resistance in NASH coexisted with increased hepatic JNK activity, a key regulator of NAFLD pathogenesis, which is also upregulated in insulin-resistant mouse models (Singh et al., 2009).

Only OBE NASH had markedly elevated circulating IL6 levels, indicating that cytokine-mediated pathways do not uniformly precede hepatic insulin resistance and NASH in humans. The link between fatty acids, mitochondrial uncoupling, and systemic inflammation might be also relevant for cardiovascular NASH comorbidities (Freigang et al., 2013). Not only cytokines, but also the hepatokine FGF21 was altered in OBE NAFL+ and even more in NASH. Serum FGF21 correlated positively with hepatic insulin resistance, supporting the contention that FGF21 may serve as biomarker of increased hepatic lipid availability associated with hepatic insulin resistance (Chavez et al., 2009). One might speculate that FGF21 could also reflect lower hepatic respiration of NASH, in view of the data linking FGF21 to impaired muscle mitochondrial respiration (Suomalainen et al., 2011).

This study benefits from simultaneous assessment of multiple features of mitochondria in intensively phenotyped volunteers, but its cross-sectional design does not allow conclusions on causal relationships. Furthermore, although there is currently no gold standard for relating mitochondrial function to mitochondrial content, the present study revealed similar results when expressing respiration rates either per protein or additionally by CSA. Finally, although NASH patients with diabetes displayed good metabolic control and comparable mitochondrial features as other NASH patients, an effect of chronic moderate hyperglycemia on the observed mitochondrial abnormalities cannot be excluded.

Taken together, we propose that increased lipid availability in the liver of obese humans with and without steatosis stimulates hepatic mitochondrial capacity and thereby serves to protect against NAFLD progression. However, augmented respiration may not be bioenergetically efficient due to leaking mitochondria and thereby promote excessive hepatic oxidative stress, challenging hepatocellular anti-oxidant defense mechanisms. Once these mechanisms fail, mitochondrial functionality decreases and hepatic insulin resistance, NAFLD progression to NASH, and systemic inflammation develop. In conclusion, these data suggest an adaptation of hepatic mitochondria in obese humans without NASH. This "hepatic mitochondrial flexibility" associated with early stages of human obesity could serve as future target for the prevention and treatment of NAFLD.

EXPERIMENTAL PROCEDURES

Study Participants

We examined 53 volunteers: 41 obese undergoing bariatric surgery (OBE) and 12 healthy lean undergoing elective abdominal surgery such as cholecystectomy or herniotomy (CON) (registered clinical trial, NCT01477957). Obese patients had stable body weight and were instructed not to follow a hypocaloric diet before surgery to avoid confounding effects on hepatic mitochondria. OBE were further classified into obese with steatosis (OBE NAFL+), without steatosis (OBE NAFL−), and NASH (OBE NASH) based on liver histology. NASH was defined by a composite outcome of steatosis, lobular inflammation, and hepatocellular injury based on a validated histological NAFLD activity score of ≥ 5 (Kleiner et al., 2005). All NASH patients displayed profound steatosis, 5 out of 7 lobular inflammation, 3 out of 7 signs of liver cell ballooning, and 6 out of 7 signs of periportal fibrosis. NASH group included two males and one female with well-controlled T2DM (mean HbA1c 7.5%), whose oral glucose-lowering medication was replaced by insulin at least 1 week before study participation. Before inclusion, all participants gave written informed consent to the protocol, which was approved by the institutional review board (IRB) of Heinrich-Heine-University Düsseldorf and conducted according to World's Medical Association Declaration of Helsinki.

Metabolic Characterization

Hyperinsulinemic-euglycemic clamps were combined with [6,6-$^2H_2$]glucose to assess whole-body and hepatic insulin sensitivity (Szendroedi et al., 2007). During the 180 min clamp (insulin 40 mU/m^2 body surface area/min, Insuman Rapid, Sanofi-Aventis Deutschland), blood glucose was kept at 90 mg/dl by periodically adjusting the 20% glucose infusion (2% enriched with [6,6-$^2H_2$] glucose).

Metabolic flexibility was assessed from standardized indirect calorimetry with a computerized gas analyzer system (Vmax Encore 29n), as the change in respiratory quotient from fasted to insulin-stimulated state. Whole-body insulin-mediated glucose disposal (M value, Rd) and endogenous glucose production (EGP) were calculated as described (Szendroedi et al., 2009). Hepatic insulin sensitivity was assessed as insulin-stimulated EGP suppression (%).

Liver Biopsies

Biopsies were taken from the lower part of right liver lobe (200–700 mg tissue) by surgeons 30 min after induction of anesthesia according to standardized protocols. Approximately 50 mg was transferred into ice-cold preservation medium (BIOPS solution) for HRR, 200 mg were placed into mitochondrial

isolation buffer (MiBO6) for mitochondrial isolation, 100 mg were fixed in 1% formaldehyde for histological examination, and remaining tissue was rapidly snap-frozen in liquid nitrogen and stored at −80°C. Liver histology was performed by an experienced hepatopathologist, according to standard techniques using hematoxylin-eosin and Masson's trichrome (Figure S2).

Mitochondrial Content and Function

Mitochondrial content was assessed from CSA and mtDNA (Benard et al., 2006; Chiappini et al., 2006). Mitochondria were isolated by differential centrifugation (Frezza et al., 2007). O_2 flux rates were measured with HRR in liver tissue and isolated mitochondria (Oroboros oxygraphs, Innsbruck) upon sequential exposure to mitochondrial substrates and titrating ADP concentrations (Kuznetsov et al., 2002) and adjusted to individual CSA values to account for mitochondrial content, in line with the majority of studies in this field (Mantena et al., 2009; Pérez-Carreras et al., 2003; Satapati et al., 2012) (for methods see Supplemental Experimental Procedures). Isolated mitochondria were also exposed to oligomycin in a separate protocol to assess leaking respiration (Wikstrom et al., 2012). Respiratory control ratio (RCR) was defined as the ratio of state 3 respiration to state o (oligomycin). Leak control ratio (LCR) was defined as the ratio of state o to state u (after fccp).

Immunoblotting

ETC complex expression was quantified by SDS-PAGE in isolated mitochondria (Mitosciences). 50 μg of mitochondrial protein were loaded per well and β-actin (Cell Signaling Technology) was used to correct for loading control. Data are expressed as ratio of signal of antibodies against ETC complexes to antibodies against β-actin. Hepatic p-JNK Thr183/Tyr185 and CHOP were quantified using specific antibodies (Cell Signaling Technology), and GADPH expression was used for loading control.

Oxidative Stress

TBARS were measured in serum and liver (Jelenik et al., 2014). Amplex Red (Molecular Probes) was used to measure hepatic H_2O_2 emission, reflecting ROS production from complexes I and III after stimulation with succinate and antimycin-A (Starkov, 2010). Catalase activity (Cayman Chemical Company) and 8-OH-dG (ELISA, Trevigen) were measured in hepatic tissue lysates.

Gene Expression Analyses

Relative quantification of hepatic mRNA expression of genes related to mitochondrial biogenesis (*TFAM*, *PGC-1a*, *NRF-1*) was assessed with real-time PCR (QuantiTect Reverse Transcription Kit, QIAGEN) and the comparative threshold cycle method (ΔΔCt) with 18S ribosomal RNA as reference gene.

Statistical Analysis

Data are presented as means ± SEM. Comparisons were performed with one-way ANOVA and correlations were analyzed with Spearman correlation. Differences were considered statistically significant at $p < 0.05$. All analyses were performed with SPSS 19.0.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.04.004.

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